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Different Populations of Parvalbumin- and Calbindin-D28k-Immunoreactive Neurons Contain GABA and Accumulate ^{3}H-D-Aspartate in the Dorsal Horn of the Rat Spinal Cord

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ABSTRACT

The colocalization of parvalbumin (PV), calbindin-D28k (CaBP), GABA immunoreactivities, and the ability to accumulate ^{3}H-D-aspartate selectively were investigated in neurons of laminae I–IV of the dorsal horn of the rat spinal cord. Following injection of ^{3}H-D-aspartate into the basal dorsal horn (laminae IV–VI), perikarya selectively accumulating ^{3}H-D-aspartate were detected in araldite embedded semithin sections by autoradiography, and consecutive semithin sections were treated to reveal PV, CaBP and GABA by postembedding immunocytochemistry. Perikarya accumulating ^{3}H-D-aspartate were found exclusively in laminae I–III, and no labelled somata were found in deeper layers or in the intermediolateral column although the labelled amino acid clearly spread to these regions. More than half of the labelled cells were localized in lamina II. In this layer, 16.4% of ^{3}H-D-aspartate-labelled perikarya were also stained for CaBP. In contrast to CaBP, PV or GABA was never detected in neurons accumulating ^{3}H-D-aspartate. A high proportion of PV-immunoreactive perikarya were also stained for GABA in laminae II and III (70.0% and 61.2% respectively). However, the majority of CaBP-immunoreactive perikarya were GABA-negative. GABA-immunoreactivity was found in less than 2% of the total population of cells stained for CaBP in laminae I–IV. A significant proportion of the GABA-negative but PV-immunoreactive neurons also showed CaBP-immunoreactivity in laminae II and IV. These results show that out of the two calcium-binding proteins, CaBP is a characteristic protein of a small subpopulation of neurons using excitatory amino acids and PV is a characteristic protein of a subpopulation of neurons utilizing GABA as a transmitter. However, both proteins are present in additional subgroups of neurons, and neuronal populations using inhibitory or excitatory amino acid transmitters are heterogeneous with regard to their content of calcium-binding proteins in the dorsal horn of the rat spinal cord.

Key words: calcium binding proteins, amino acid transmitters, immunocytochemistry, autoradiography

The presence of two calcium-binding proteins, parvalbumin (PV) and calbindin-D28k (CaBP) in the central nervous system has received increasing interest in recent years. Specific and significant changes in calbindin gene expression and in the number of neurons containing either PV or CaBP indicate that they are involved in molecular events accompanying aging (Iacopino and Christakos, '90), seizure activity (Kamphuis et al., '89; Sloviter, '89), and various neurodegenerative diseases (Ichimiya et al., '89; Iacopino and Christakos, '90). In neuroanatomical studies, the immunocytochemical detection of these proteins provides an excellent tool to study the morphology and neurochemical character of specific subsets of neurons. Although PV and CaBP have been localized in the same neuron types in some

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sites of the central nervous system, e.g., Purkinje cells of the cerebellum or periglomerular cells of the olfactory bulb (Celio and Heizmann, '81; Baimbridge and Miller, '82), the two proteins generally appear to be present in different subclasses of neurons. In the cat and monkey cerebral cortex, PV and CaBP immunoreactivities have been found in functionally and morphologically separate subpopulations of GABAergic neurons (Celio, '86; Demeulemeester et al., '89; Hendry et al., '89; van Brederode et al., '90). In the rat hippocampus PV is localized preferentially in fast spiking GABAergic interneurons (Kawaguchi et al., '87; Kosaka et al., '87), while CaBP-immunoreactivity is found in a distinct population of interneurons and in pyramidal cells of the CA1 region (Baimbridge and Miller, '82; Baimbridge et al., '82). Septohippocampal GABAergic projection neurons terminating on GABAergic interneurons in the hippocampus (Freund and Antal, '88) have also been found to be PV-immunoreactive (Freund, '89). Both PV and CaBP have been demonstrated in cells that contain high levels of cytochrome oxidase activity in the visual system of zebra finch (Braun et al., '85) and in dorsal root ganglia of rat (Carr et al., '89). Neurons displaying immunoreactivity for CaBP have been identified as cells of origin of primary afferents innervating skeletal muscles in the chick (Philippe and Droz, '89).

Calcium-binding proteins are also present in the spinal cord (Celio and Heizmann, '81; Garcia-Segura et al., '84; Fournet et al., '86; Yamamoto et al., '89). In a recent paper we have demonstrated that, similar to other brain regions, PV- and CaBP-immunoreactivities are displayed by distinct populations of spinal interneurons (Antal et al., '90a). The aim of the experiments presented here was to characterize the putative transmitters of these specific subgroups of interneurons. Using autoradiography and postembedding immunocytochemical methods we have studied the coexistence of these calcium-binding proteins with GABA and the ability to selectively accumulate ^{3}H-D-aspartate in laminae I–IV of the dorsal horn of the rat spinal cord.

Preliminary observations from this experiment have been reported in abstract form (Antal et al., '90b).

MATERIALS AND METHODS

Animals, injection of ^{3}H-D-aspartate, and preparation of tissue sections

Experiments were carried out on two Wistar Kyoto rats of 250–350 g body weight. Animals were anaesthetised with an intraperitoneal injection of a mixture of 60 mg/kg sodium pentobarbitone and 100 mg/kg chloral hydrate, and paralysed with suxamethonium chloride (4 mg/kg) applied through a cannula introduced into the femoral artery. A laminectomy was performed on the thoracic spinal cord (T9-T10 segments), while the animal was held in a stereotaxic frame. One side of the cord was injected with 4.5 mM ^{3}H-D-aspartate (Amersham, 22 Ci/mmol, dried and redissolved in artificial cerebrospinal fluid). The coordinates for the injections were 0.6–0.7 mm from the midline and 0.6–0.8 mm from the dorsal surface of the cord. In each animal, four separate sites distributed rostro-caudally (0.5 mm apart) were injected. Each site received about 10 μCi in 100 nl (one animal), or 20 μCi in 200 nl (one animal) of isotope in 5–20 nl steps over 15 min through a glass micropipette (tip diameter 30–50 μm).

Twenty-four hours after the injections the animals were perfused transcardially with Tyrode's solution (oxygenated with a mixture of 95% O_2, 5% CO_2), followed by a fixative containing 3% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The lower thoracic and upper lumbar spinal cord was removed and postfixed in the same fixative for 1 hour. Tissue blocks were sectioned at 60 μm on a vibratome. Following several washes in 0.1 M PB, sections were treated with 1% OsO_4 dissolved in 0.1 M PB for 30–60 minutes, then dehydrated and flat-embedded in epoxy resin (Durcupan, Fluka) on glass slides. Selected sections were reembedded, then serial semithin sections were cut at 0.5 μm and dried on a hot plate onto slides coated with chrome alum-gelatin. Consecutive sections were reacted to visualize PV, CaBP, and GABA immunoreactivity by postembedding immunocytochemistry, or ^{3}H-D-aspartate by autoradiography.

Autoradiography

After dipping in Ilford K5 emulsion, sections were exposed for a period of time ranging from 3 days to 3 weeks in the dark at 4°C. Autoradiograms were developed with Kodak D19 developer and counterstained with toluidine blue.

Immunocytochemistry

Parvalbumin, calbindin, and GABA immunoreactivities were detected on consecutive semithin sections using the method described by Somogyi and Hodgson ('85). The resin was etched by using ethanolic sodium hydroxide for 30 minutes, then the sections were transferred to 1% sodium metaperiodate for 10 minutes. Sections were treated with 20% normal goat serum (Vector Labs.) for 20 minutes, then incubated with either anti-PV (diluted 1:400), anti-CaBP (diluted 1:1000) or anti-GABA (diluted 1:1000) antisera for 2 hours. All antisera were raised in rabbit. The immunological and immunocytochemical characteristics of anti-PV (Kägi et al., '87), anti-CaBP (Baimbridge et al., '82; Sloviter, '89) and anti-GABA antibodies (Hodgson et al., '85) have been published earlier. Subsequently goat anti-rabbit IgG (ICN, diluted 1:50) was layered over the slides for 40–50 minutes, followed by rabbit-PAP (DAKO, diluted 1:100) for 1 hour. Antibodies were diluted in Tris (10 mM)-phosphate (10 mM) buffered isotonic saline (TPBS, pH 7.4), to which 1% normal goat serum and 0.1% Tween 20 were added. Immunoreactions were visualized with diaminobenzidine chromogen and the reaction endproduct was intensified with OsO_4.

To check the specificity of the immunostaining method, sections were treated by the immunocytochemical procedure described above with the primary antisera omitted or replaced with normal rabbit serum (diluted 1:100). No specific staining was observed in these sections.

To demonstrate the colocalization of different substances in the same cell, we compared adjacent sections treated in different ways and identified the same perikarya sectioned into consecutive sections. Cell numbers and quantitative data for the presence of coexistence are based upon studying 30 sets of consecutive section pairs from each combination.

RESULTS

^{3}H-D-aspartate uptake

Injection sites. The injection sites were located over the basal part of the dorsal horn. The injected regions were almost identical at the four injection sites in the same

animal. In one animal (animal 1) the injection was centered in the medial half of laminae IV–VI, while the lateral third of the same laminae and the adjacent lateral white matter were involved in the other rat (animal 2).

Distribution of perikarya accumulating ^{3}H-D-aspartate. Labelled perikarya were found exclusively in laminae I–III, and their distribution was very similar at the four separate injection sites in the same animal. In animal 1 the labelling was restricted to the medial two-thirds of the superficial dorsal horn (Fig. 1). In animal 2, all of the labelled perikarya were found in the lateral half of laminae I–III. Most of the perikarya accumulating ^{3}H-D-aspartate were found at the segmental level of the injection sites. Labelled cells were only occasionally encountered in sections two or three segments away from the sites of application in either the rostral or caudal directions. More than half of the labelled cells were encountered in lamina II in both animals (Table 1). In contrast, the relative density of labelled perikarya in laminae I and III was significantly different in the two animals. In addition to lamina II, the labelling in animal 1 was mainly concentrated in lamina III. In animal 2, however, the number of labelled perikarya was considerably higher in lamina I than in lamina III (Table 1). In addition to perikarya, a prominent terminal labelling restricted to the medial third of laminae II and III was also present in animal 1 (Fig. 1).

Distribution of PV-, CaBP-, and GABA-immunoreactivity in laminae I–IV

Previous studies have demonstrated immunoreactivity to PV, CaBP (Yamamoto et al., '89; Antal et al., '90a), and GABA (Magoul et al., '87; Todd and McKenzie, '89; Todd and Sullivan, '90) in the spinal cord of the rat, and the present findings are in general agreement with these reports.

PV-immunoreactivity. PV-positive cell bodies were revealed in laminae II–IV, however, lamina I was practically devoid of labelling (Fig. 2a, Table 2). Of the 155 PV-immunoreactive perikarya counted, only one faintly stained cell was revealed in lamina I. Confirming our previous finding (Antal et al., '90a), the inner layer of lamina II was densely packed with immunoreactive punctate profiles (Fig. 2a).

CaBP-immunoreactivity. The density of immunostaining obtained for CaBP surpassed PV-immunoreactivity. Laminae I and II were darkly stained with punctate profiles and CaBP-positive perikarya were found in all four laminae

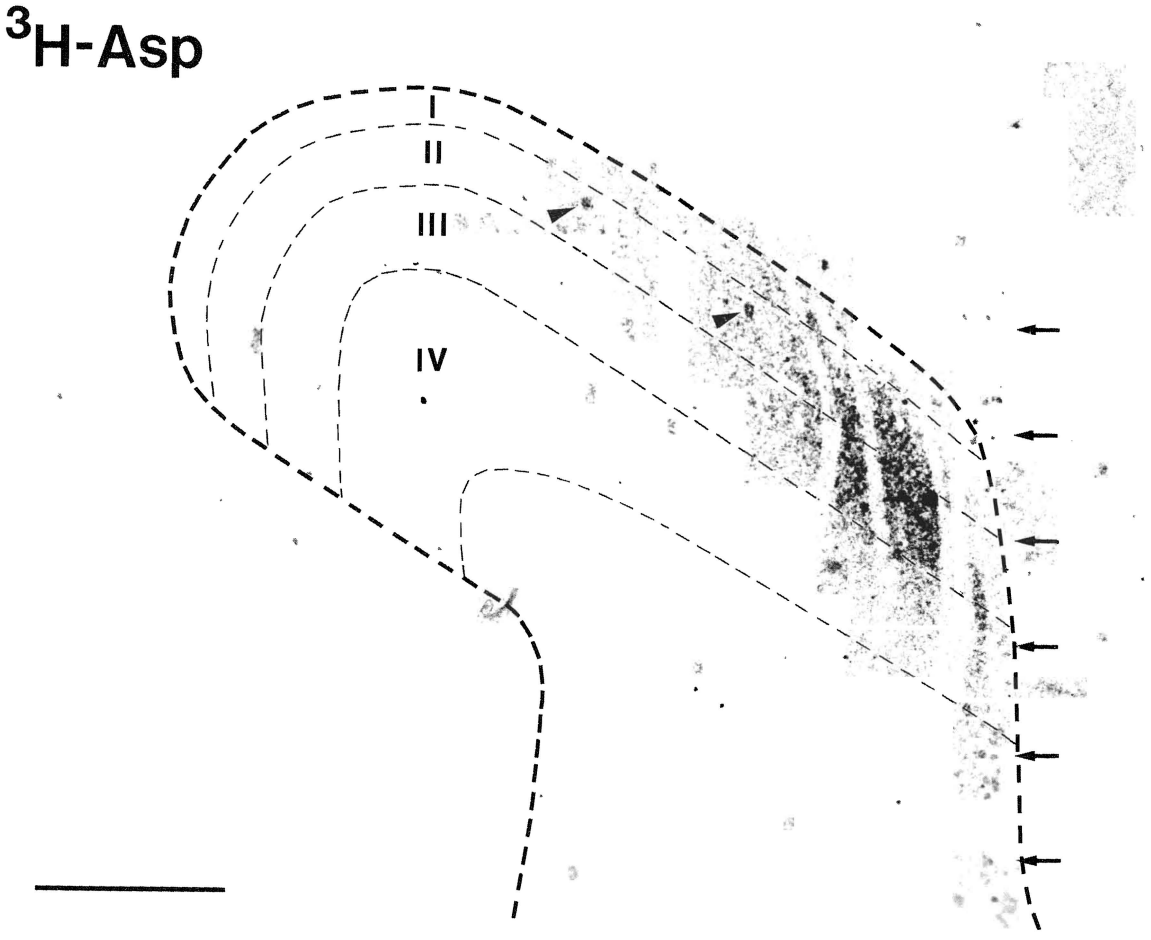

Fig. 1. Semithin section of the dorsal horn processed for autoradiography. The section is taken from the immediate neighbourhood of an injection site of ^{3}H-D-aspartate. The track of the cannula is partly seen and indicated by arrows. Exposition time is 14 days. The borders of Rexed laminae I–IV and between the gray and white matters are drawn with dashed lines. Arrowheads point to neuronal perikarya accumulating ^{3}H-D-aspartate in lamina II. Note the high density of silver grains in the neuropile at the medial third of laminae II–III. Bar = 100 μm.

TABLE 1. Number of Perikarya Accumulating ^{3}H-D-Aspartate in Laminae I–III[1]

	Lamina I	Lamina II	Lamina III	Total
Animal 1	74	237	125	436
Animal 2	80	171	34	285

[1]According to current hypothesis only those neurons which had their axon and axon terminals in the injected zone became labelled. Therefore this exogenously introduced marker would not label every cell that has the molecular mechanism for the selective uptake of acidic amino acids.

studied (Fig. 2b, Table 2). Most of the neurons expressing positive immunoreaction for CaBP were found in lamina II, while they were more sparsely distributed in laminae I, III, and IV (Fig. 2b, Table 2).

GABA-immunoreactivity. Immunoreactive cells were present throughout laminae I–IV (Fig. 2c, Table 2). More than 40% of stained perikarya were found in lamina II, while the rest of labelled cells was distributed in laminae I, III, and IV in nearly equal numbers (Table 2). A dense punctate staining was also revealed in laminae I–III (Fig. 2c).

Colocalization of substances

In the series of 30 consecutive section pairs investigated, we counted 3191 perikarya that were either immunoreactive for PV, CaBP or GABA, or accumulated ^{3}H-D-aspartate. Colocalization of different substances in various combinations was detected in 214 cell bodies. None of the perikarya expressed more than two positive reactions.

Coexistence of PV- and GABA-immunoreactivities. Most of perikarya displaying immunoreactivity for both PV and GABA were found in laminae II and III (Fig. 3, Table 2). In these laminae, 70.0% and 61.2% of the total population of PV-immunoreactive cell bodies were also stained for GABA (Table 3). The proportion of coexistence was considerably lower in lamina IV (14.9%), and the single PV-immunoreactive perikaryon found in lamina I did not show GABA-immunoreactivity. Since the number of cell bodies immunoreactive for GABA surpassed the number of PV-positive ones, double-labelled cells represented a considerably lower proportion of GABA-immunoreactive perikarya. This value varied between 21.0% and 5.0% in the different laminae (Table 5).

Coexistence of CaBP- and GABA-immunoreactivities. Perikarya immunostained for both CaBP and GABA were only occasionally seen (Fig. 4 Table 2). The frequency of occurence of double-labelled cells was very similar in the different layers, and represented 1.1–3.2% of CaBP-immunoreactive and 0.7%–6.1% of GABA-immunoreactive perikarya (Tables 4,5).

Coexistence of PV- and CaBP-immunoreactivities. The frequency of occurence of perikarya displaying positive immunoreaction for both PV and CaBP varied in a wide range in the different laminae. High proportions of PV-positive cells were immunoreactive also for CaBP in laminae II and IV (27.5% and 38.3% respectively), however, only 5.9% of them showed positive staining for CaBP in lamina III (Table 3). CaBP was detected also in the one PV-positive cell in lamina I (Figs. 5b,d). Since the number of cell bodies immunoreactive for CaBP surpassed the number of PV-positive ones, double-labelled cells represented a lower proportion of CaBP-immunoreactive perikarya. This value did not exceed 1.1% in laminae I–III (Table 4). PV-immunoreactivity was found in a relatively high proportion of CaBP-positive perikarya only in lamina IV, where nearly one-fifth of CaBP-immunoreactive cells were also stained for PV (Table 4). The double-labelled cells usually showed moderate PV and strong CaBP immunoreactivity (Fig. 5).

Coexistence of CaBP-immunoreactivity and selective uptake of ^{3}H-D-aspartate. The distribution and proportion of double-labelled cells were very similar in the two animals receiving injections of ^{3}H-D-aspartate into the medial or lateral portion of the basal dorsal horn. Most of perikarya accumulating ^{3}H-D-aspartate and displaying positive immunoreaction for CaBP were found in lamina II in both animals (Fig. 6). One-sixth (16.4%) of the perikarya accumulating ^{3}H-D-aspartate were also labelled for CaBP in this lamina. The proportion of double-labelled cells was more moderate in lamina III (5.0%), while lamina I was almost completely devoid of double-labelled perikarya (Tables 2, 4).

None of the cells accumulating ^{3}H-D-aspartate displayed positive immunoreaction for either PV or GABA.

DISCUSSION

Transfer of ^{3}H-D-aspartate from the injection site to labelled perikarya

In principle, different processes could be involved in the transfer of ^{3}H-D-aspartate from the injection site into the labelled neuronal perikarya: (i) The non-selective local uptake of aspartate by somata and dendrites around the injection site is one possibility. It is highly probable that this mechanism did not play a considerable role in our experiment, since the pattern of perikaryal labelling can hardly be explained by this mechanism. No labelled perikaryon was seen in lamina IV, although this lamina was located closest to the injection site. The large number of labelled neurons in lamina II and the relatively low occurrence of them in lamina III are also inconsistent with the uptake from a diffusion gradient. Furthermore, more than twice as many neurons were counted in lamina I as in lamina III when the ^{3}H-D-aspartate was delivered into the lateral portion of the basal dorsal horn. (ii) The ^{3}H-D-aspartate could be transported by axons of neurons projecting from the site of injection to the superficial dorsal horn, and subsequently transferred transneuronally into perikarya located in laminae I–III. This interpretation is also unlikely, since neurons that fulfill the anatomical requirements of such a transport have never been reported in the basal dorsal horn. (iii) The most conceivable interpretation of the transfer is an uptake by axon terminals or less likely by fibres of passage (Streit, '80; Cuenod and Streit, '83) at the injection site, followed by retrograde axonal transport to the cells of origin. The chemical specificity of this presumably high affinity amino acid uptake system has been tested with a wide range of compounds (Balcar and Johnston, '72; Beart et al., '72; Davies and Johnston, '76; Streit, '80). Aspartate and glutamate are transported by the same uptake process, which is not shared by glycine or GABA (Balcar and Johnston, '72; Logan and Snyder, '71; Streit, '80; Rustioni and Cuenod, '82). These earlier data as well as our finding of a specific distribution of labelled perikarya suggest that ^{3}H-D-aspartate has been accumulated in neurons utilizing excitatory amino acids in the dorsal horn. Pharmacological and biochemical studies confirming the importance of excitatory amino acids in neurotransmission in the spinal cord support this conclusion (Davidoff et al., '67; Graham et al., '67; Duggan,

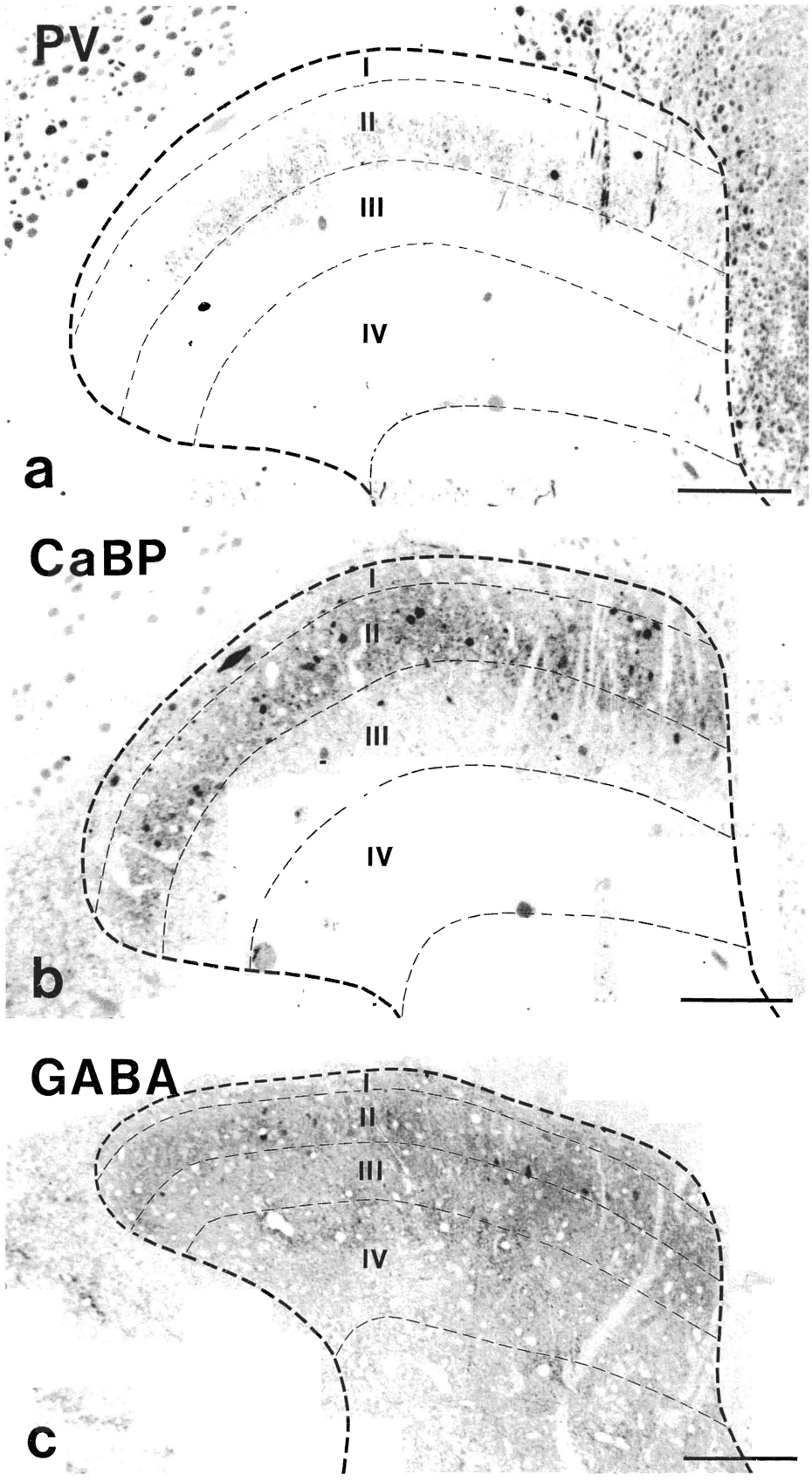

Fig. 2. Semithin sections of the dorsal horn immunostained for PV (**a**), CaBP (**b**), and GABA (**c**). The borders of Rexed laminae I–IV and between the gray and white matters are drawn by dashed lines. Bars = 100 μm.

'74; Berger et al., '77; Salt and Hill, '83; Miller et al., '88; Skilling et al., '88).

Neurons accumulating ^{3}H-D-aspartate

This study confirms a previous report of Rustioni and Cuenod ('82) that neurons accumulating exogenously applied ^{3}H-D-aspartate are located in the superficial layers of the dorsal horn.

A population of labelled neurons presumably comprises projection neurons having extensive local axon collaterals in the basal dorsal horn. Cells of origin of the spinothalamic projection (Giesler et al., '79; Granum, '86; Lima and Coimbra, '88; Hylden et al., '89), spinomesencephalic tract (Menétrey et al., '82; Liu, '83; Hylden et al., '86), spinoreticular fibres (Menétrey et al., '83; Lima, '90; Lima and Coimbra, '90), spinocervical fibres (Giesler et al., '78), and postsynaptic dorsal column system (Giesler et al., '84) are located in the dorsal horn of the rat spinal cord. In view of their segmental course, all of these pathways can be involved in the labelling. The axons of these neurons run through the sites of aspartate injection, where many of them have extensive local axon collaterals (Brown, '81; Brown and Fyffe, '81).

One of the most striking findings of this experiment is the large number of labelled perikarya revealed in lamina II. Neurons in lamina II also project to the thalamus (Granum,

TABLE 2. Number of Perikarya Expressing One or Two Molecular Markers in Laminae I-IV

	No. of Labelled Neurons				
Neuronal Marker	Lamina I	Lamina II	Lamina III	Lamina IV	Total
PV	1	40	67	47	155
CaBP	154	1020	370	91	1635
GABA	124	335	195	140	794
^{3}H-D-Asp	154	408	159	—	721
PV + GABA	—	28	41	7	76
CaBP + GABA	3	12	12	1	28
PV + CaBP	1	11	4	18	34
^{3}H-D-Asp + CaBP	1	67	8	—	76

TABLE 3. Proportion of PV-Immunoreactive Neurons Displaying Immunoreactivity for Either GABA or CaBP in Laminae I–IV

	Lamina I	Lamina II	Lamina III	Lamina IV
$\frac{\text{PV + GABA}}{\text{PV}} \times 100$	—	70.0%	61.2%	14.9%
$\frac{\text{PV + CaBP}}{\text{PV}} \times 100$	100.0%	27.5%	5.9%	38.3%
$\frac{\text{PV without CaBP or GABA}}{\text{PV}} \times 100$	—	2.5%	32.9%	46.8%
	100.0%	100.0%	100.0%	100.0%

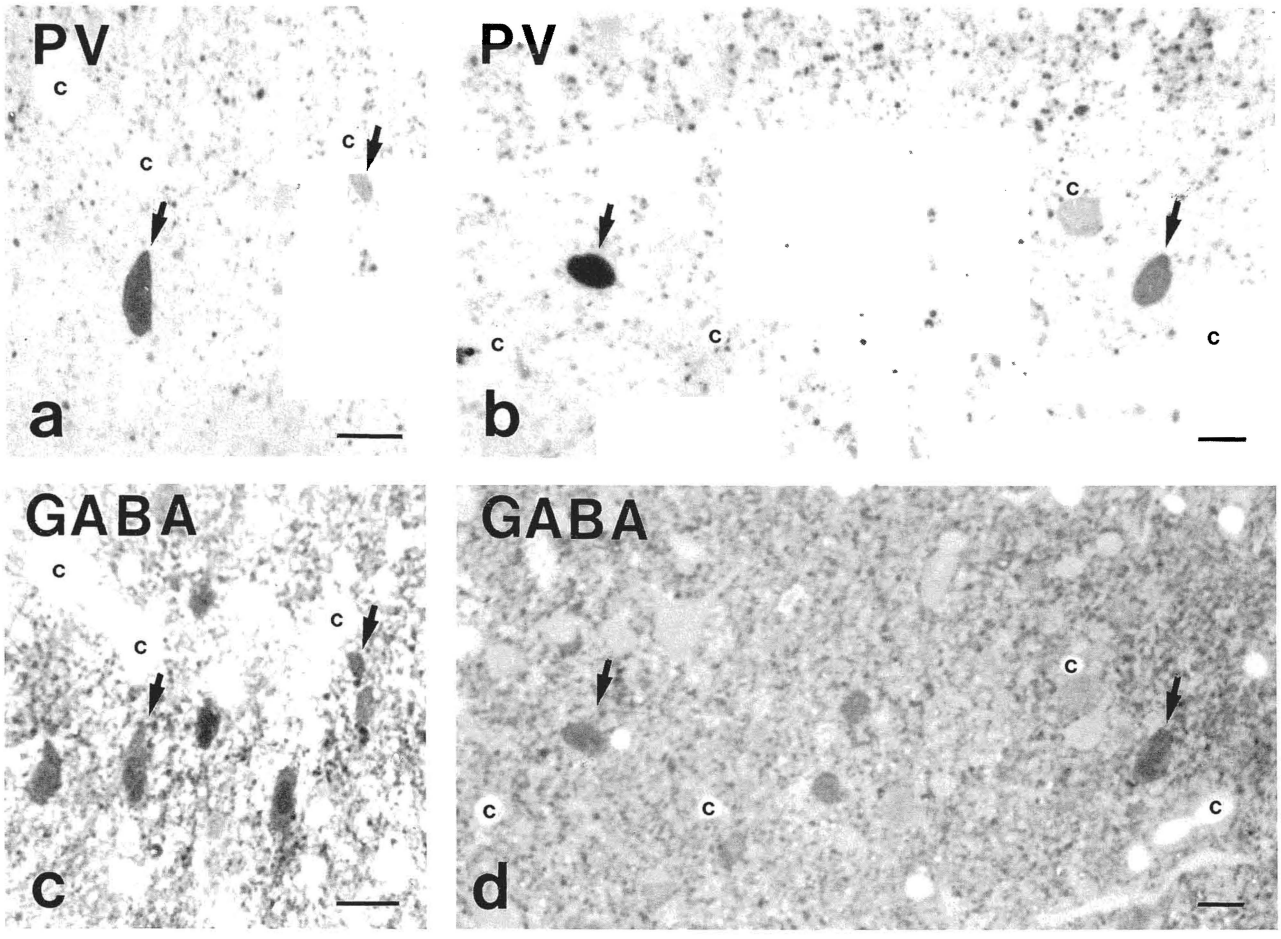

Fig. 3. Micrographs from consecutive semithin sections immunostained for either PV or GABA. Areas of lamina II (**a,c**) and lamina III (**b,d**) are shown. Arrows point to perikarya displaying immunoreactivity for both PV and GABA. c: capillaries serving as landmarks in fitting the micrographs. Bars = 10 μm.

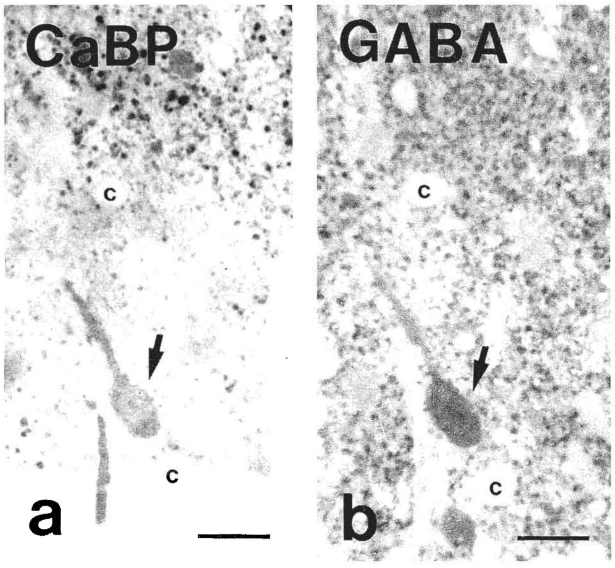

Fig. 4. Micrographs from consecutive semithin sections immunostained for either CaBP or GABA. The same area of lamina III is shown. Arrows point to a perikaryon displaying immunoreactivity for both CaBP and GABA. c: capillaries serving as landmarks in fitting the micrographs. Bars = 10 μm.

TABLE 4. Frequency of Occurrence of CaBP Immunoreactivity in Cells Accumulating ^{3}H-D-Aspartate in Laminae I–III, and Proportions of CaBP-Immunoreactive Neurons Displaying Immunoreactivity for Either GABA or PV

	Lamina I	Lamina II	Lamina III	Lamina IV
$\frac{\text{CaBP} + {}^3\text{H-Asp}}{{}^3\text{H-Asp}} \times 100$	0.6%	16.4%	5.0%	—
$\frac{\text{CaBP} + \text{GABA}}{\text{CaBP}} \times 100$	1.9%	1.1%	3.2%	1.1%
$\frac{\text{CaBP} + \text{PV}}{\text{CaBP}} \times 100$	0.6%	1.1%	1.1%	19.8%

TABLE 5. Proportion of GABA-Immunoreactive Neurons Expressing Immunoreactivity for Either PV or CaBP

	Lamina I	Lamina II	Lamina III	Lamina IV
$\frac{\text{PV} + \text{GABA}}{\text{GABA}} \times 100$	—	8.3%	21.0%	5.0%
$\frac{\text{CaBP} + \text{GABA}}{\text{GABA}} \times 100$	2.4%	3.6%	6.1%	0.7%
$\frac{\text{GABA without PV or CaBP}}{\text{GABA}} \times 100$	97.6%	88.1%	72.9%	94.3%
	100.0%	100.0%	100.0%	100.0%

'86), mesencephalon (Liu, '83), lateral cervical nucleus (Giesler et al., '78), and dorsal reticular nucleus in the lower brain stem (Lima, '90), but their very moderate contribution to these pathways cannot explain the strong labelling revealed in lamina II. The majority of labelled perikarya in lamina II presumably represent local interneurons. In their developmental study, Beal ('83) and Bicknell and Beal ('84) found that a proportion of islet cells in lamina II develop short, intrinsic axon collaterals that ramify within laminae III–IV. Axons of neurons that cannot be classified as either islet or stalked cells may also course ventrally (Todd and Lewis, '86). Intracellular injections of HRP into neurons located in lamina II have also revealed axon-like processes dipping down to lamina IV (Woolf and Fitzgerald, '83). These reports and the large number of perikarya accumulating ^{3}H-D-aspartate in lamina II suggest that a considerably higher proportion of neurons located in lamina II may possess axons arborizing in laminae III–IV than previously thought.

No perikaryon accumulating ^{3}H-D-aspartate has been found in lamina IV. This finding is a strong argument in favour of the specificity of the radiolabelling, and indicates that neurons in lamina IV presumably do not utilize excitatory amino acids as neurotransmitters. The other possible explanation of this negative finding, namely that the axons of neurons located in lamina IV do not terminate in the sites of injections, is less likely since extensive axon collaterals of lamina IV neurons have been demonstrated in the basal dorsal horn (Brown, '81; Brown and Fyffe, '81).

Colocalization of PV-, CaBP-, GABA-immunoreactivities and selective uptake of ^{3}H-D-aspartate in laminae I–IV

Lamina I. Confirming our previous finding (Antal et al., '90a), with the exception of one faintly labelled perikaryon, PV-immunoreactivity has not been revealed in lamina I. On the other hand, neurons displaying CaBP or GABA immunoreactivity, or accumulating ^{3}H-D-aspartate, have been frequently encountered in this lamina. Despite the large number of stained perikarya, double-labelled neurons have not been found to a notable extent; rather three distinct populations of neurons, each with a separate molecular marker, were labelled in lamina I.

The dendritic and axonal arbor of interneurons in lamina I are generally confined to the marginal zone and do not extend beyond lamina II (Beal et al., '81; Bennett et al., '81; Lima and Coimbra, '86). These observations suggest that perikarya labelled with ^{3}H-D-aspartate represent projection neurons in lamina I. They may be part of the spinoreticular, spinomesencephalic and spinocervical projection systems (Giesler et al., '78; Hylden et al., '86, '89; Leah et al., '88; Lima and Coimbra, '89, '90), and presumably possess deep local axon collaterals accounting for the retrograde labelling.

Only one of the 154 ^{3}H-D-aspartate-labelled neurons showed CaBP immunoreactivity in lamina I. In a recent paper we demonstrated (Antal et al., '90a), that the CaBP-immunoreactive neurons in lamina I resemble fusiform, pyramidal and flattened neurons described by Lima and Coimbra ('86). The retrograde labelling of such perikarya after thalamic, mesencephalic and bulbar injections of HRP or cholera toxin (Menétrey et al., '82; Lima and Coimbra, '88, '89, '90; Hylden et al., '89) suggests that CaBP-immunoreactive cells may also comprise projection neurons. The low proportion of colocalization of GABA and CaBP in lamina I indicates that the majority of these neurons are also excitatory. Furthermore, the low level of coexistence between the specific uptake of ^{3}H-D-aspartate and CaBP-immunoreactivity suggests that CaBP-immunoreactive cells in lamina I either use transmitters distinct from excitatory amino acids or do not have local axon collaterals at the sites of ^{3}H-D-aspartate injections. Spinothalamic neurons containing dynorphin or vasoactive intestinal polypeptide (Leah et al., '88) may be a part of this cell population.

Lamina II. On the basis of morphological characteristics, CaBP-immunoreactive cells were interpreted as belonging to the so called "atypical" class of neurons in lamina II

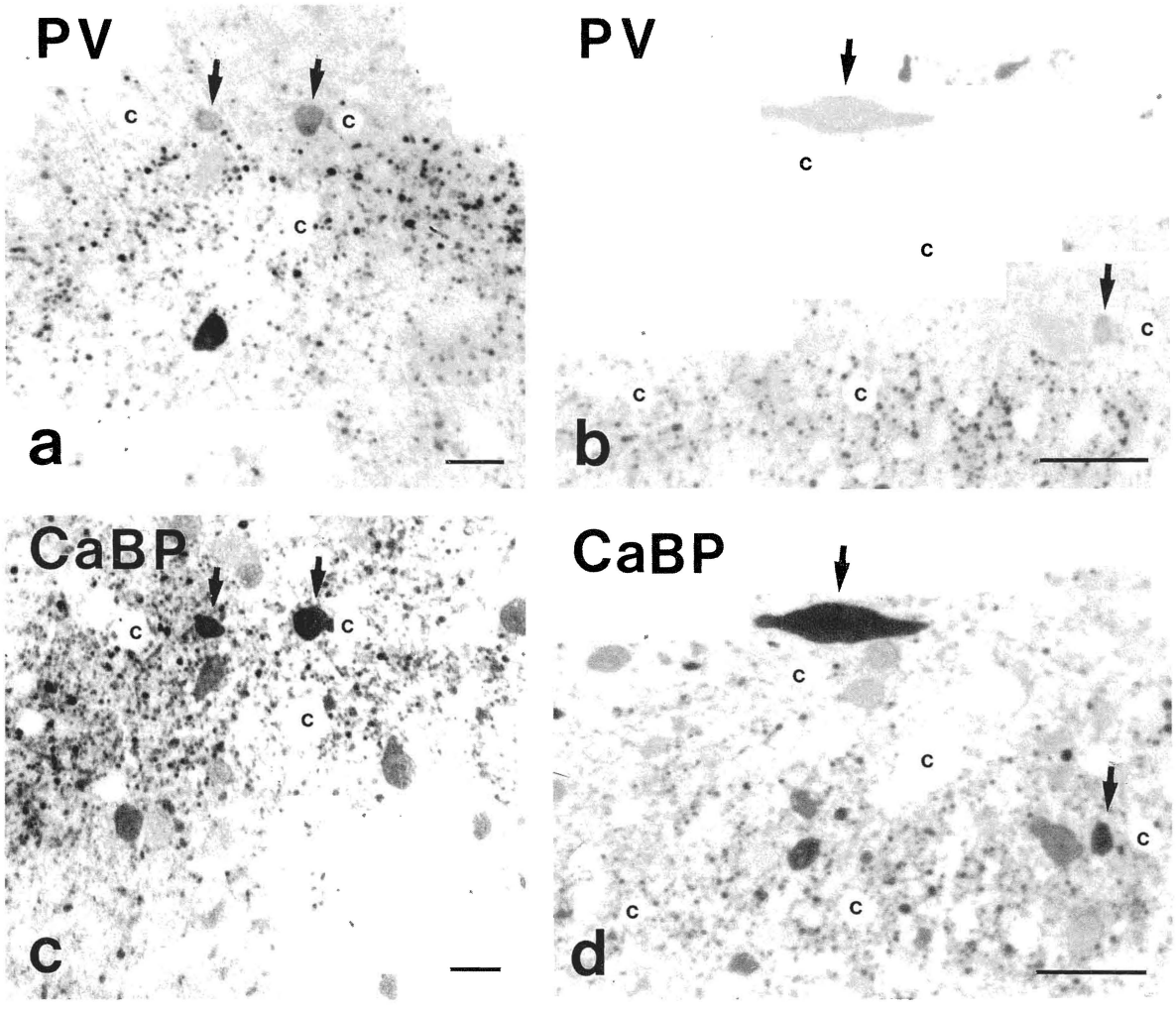

Fig. 5. Micrographs from consecutive semithin sections immunostained for either PV or CaBP. The same areas of lamina II and III (**a,c**), and lamina I and II (**b,d**) are shown. Arrows point to perikarya displaying immunoreactivity for both PV and CaBP. c: capillaries serving as landmarks in fitting the micrographs. Bars = 10 μm.

(Antal et al., '90a). This cell type represents a large proportion of neurons; 37 of 100 Golgi-impregnated neurons in lamina II were characterized as "atypical" cells by Todd and Lewis ('86). The large number of CaBP-immunoreactive perikarya in the present study is in agreement with this finding, and suggests that CaBP is present in a significant proportion of "atypical" cells. The low percentage of colocalization between CaBP and GABA in lamina II indicates that the majority of CaBP-immunoreactive "atypical" cells are excitatory neurons. Furthermore, a proportion of them may use excitatory amino acids as neurotransmitters (16.4% of perikarya accumulating ^{3}H-D-aspartate were also labelled for CaBP in lamina II).

The location and dendritic morphology of PV-immunoreactive neurons correspond to that of islet cells (Antal et al., '90a). Many of the islet cells contain GAD (Barber et al., '82) and GABA (Todd and McKenzie, '89), and almost half of GABA-immunoreactive cells can also be immunostained for glycine (Todd and Sullivan, '90). However, a distinct class of islet cells which does not contain GABA may also exist. Todd and McKenzie ('89) recently reported that 6 of 17 Golgi-impregnated islet cells were negative for GABA. Our present results show that PV is predominantly present in the GABAergic subpopulation of islet cells. GABA-immunoreactivity has been found in 70.0% of neurons stained for PV in lamina II. Only a small fraction (2.5%) of PV-immunoreactive neurons showing neither GABA nor CaBP-immunoreactivity, may represent GABA-negative islet cells. The GABA-negative but CaBP-immunoreactive population of PV-immunoreactive neurons may belong to other morphological categories, since CaBP-positive cells in lamina II have never been found to possess the large rostro-caudally oriented dendritic trees (Yamamoto et al., '89; Antal et al., '90a) characteristic of islet cells (Gobel, '78; Todd and Lewis, '86). Neurons containing both PV and CaBP, but negative for GABA, may represent a specific subset of "atypical" neurons. The lack of ^{3}H-D-aspartate in these neurons suggests that their axons may be confined to the substantia gelatinosa, or that they may possess axons arborising in deeper laminae but do not have the uptake system for excitatory amino acid neurotransmitters. Instead of excitatory amino acids, they may contain substance P or other neuropeptides (Hunt et al., '81).

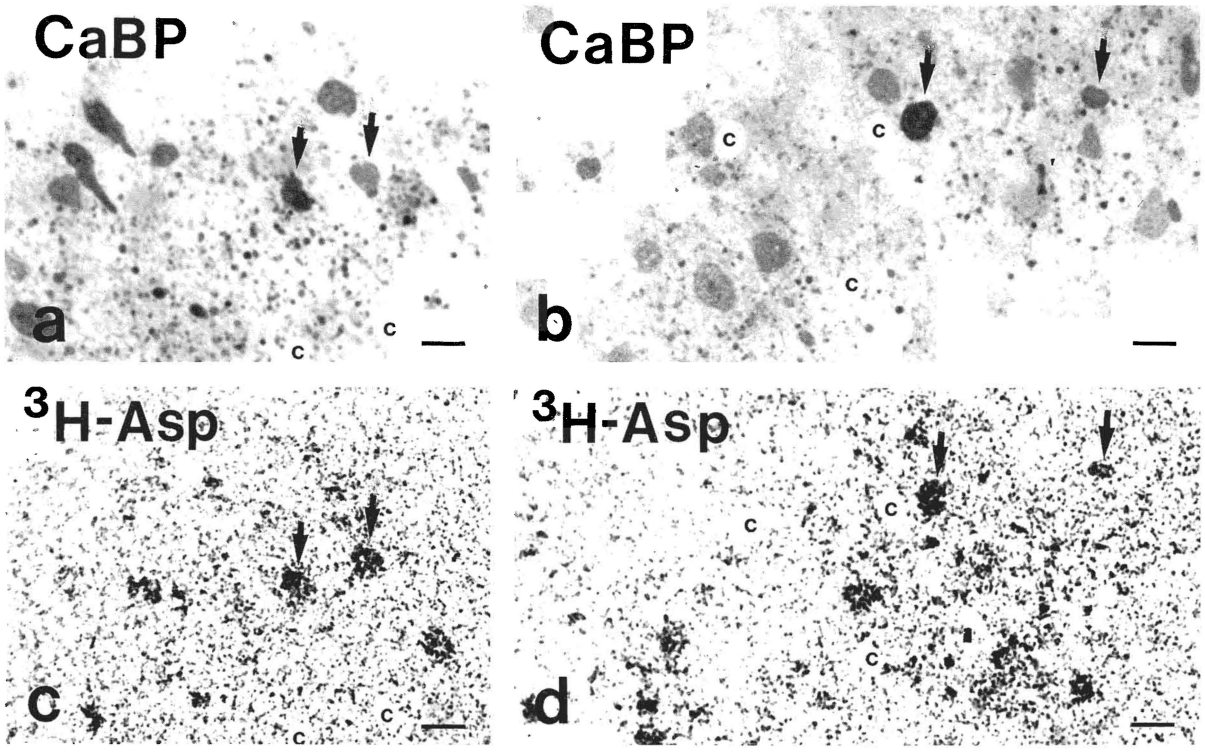

Fig. 6. Micrographs from consecutive semithin sections immunostained for CaBP or processed for autoradiography. The same areas of lamina II (**a** + **c**, **b** + **d**) are shown. Arrows point to those perikarya which show CaBP-immunoreactivity and accumulate ^{3}H-D-aspartate as well. c: capillaries serving as landmarks in fitting the micrographs. Bars = 10 μm.

Lamina III. CaBP-immunoreactive cells in lamina III are small fusiform neurons with dendrites arborizing in the rostrocaudal direction (Antal et al., '90a). The present experiment provides new data for the neurochemical characterization of this neuron population. The low level of coexistence between CaBP and GABA-immunoreactivity (6.1%), suggests that the majority of CaBP-immunoreactive fusiform cells in lamina III are excitatory neurons, and some of them may utilize excitatory amino acids as neurotransmitters (5% of perikarya accumulating ^{3}H-D-aspartate were stained also for CaBP).

On the basis of their dendritic morphology, PV-positive cells in lamina III are interpreted as pyramidal neurons (Antal et al., '90a). Pyramidal cells in lamina III probably act as inhibitory neurons (Réthelyi and Szentágothai, '69), possibly playing a role in the gate control mechanism suggested by Melzack and Wall ('65). The high degree of coexistence between PV and GABA in lamina III confirms this interpretation. Almost two-thirds of PV-positive cells also displayed GABA-immunoreactivity in lamina III, and the additional one-third may comprise neurons containing enkephalin, neurotensin or cholecystokinin (Senba et al., '82; Fuji et al., '85).

Lamina IV. Confirming our previous finding that some neurons in lamina IV contain both PV and CaBP (Antal et al., '90a), the highest proportion of coexistence between PV and CaBP has been found in this lamina. Nearly 30% of PV- and 20% of CaBP-immunoreactive cells were also immunostained for the other protein. The distribution and dendritic morphology of these neurons resemble cells of origin of the spinocervical tract (Brown, '81; Antal et al., '90a). Most of the axons composing the spinocervical tract arise from neurons in lamina IV, and the great majority of them give rise to local axon collaterals terminating in laminae IV–VI (Brown, '81). Although ascending afferents display glutamate-like immunoreactivity in the lateral cervical nucleus (Broman et al., '90), neurons accumulating ^{3}H-D-aspartate have not been revealed in lamina IV. This suggests that the spinocervical tract consists of neurochemically heterogeneous fiber populations. Neurons projecting from lamina I (Brown, '81) may contain excitatory amino acids, while neurons projecting from lamina IV may utilize other transmitters. The nearly 15% coexistence of PV and GABA in lamina IV suggests that GABAergic neurons may also be involved in this projection system.

Conclusion

The selective uptake of ^{3}H-D-aspartate by neurons located in laminae I–III confirms the results of previous biochemical, pharmacological, autoradiographic, and immunocytochemical studies, and suggests that excitatory amino acids are used as neurotransmitters in the dorsal horn of the rat spinal cord. Neurons utilizing excitatory amino acids can be projection neurons participating in spinothalamic, spinomesencephalic, spinoreticular tracts as well as local interneurons in laminae I–III.

The coexistence of the two calcium-binding proteins, parvalbumin and calbindin-D28k, with GABA and with the selective uptake of ^{3}H-D-aspartate is strikingly different. A significant population of PV-immunoreactive neurons is also immunoreactive for GABA, while 98.2% of the total population of CaBP-immunoreactive neurons are GABA-

negative in laminae I–IV. On the other hand, a population of neurons accumulating ^{3}H-D-aspartate contain CaBP, but none of them show PV-immunoreactivity. These results suggest that in the dorsal horn of the rat spinal cord neurons using excitatory or inhibitory amino acids as transmitters largely differ in their content of calcium-binding proteins.

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